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IMMUNOLOGICALLY ACTIVE POLYSACCHARIDES OF *EUPATORIUM CANNABINUM* AND *EUPATORIUM PERFOLIATUM**

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Key Word Index—*Eupatorium cannabinum*; *Eupatorium perfoliatum*; Asteraceae; 4-*O*-methylglucuronoxylans; plant polysaccharide; structure analysis; immunological activity.

Abstract—From the alkaline aqueous extract of the herbal part of *Eupatorium cannabinum* and *Eupatorium perfoliatum* two homogeneous polysaccharides (PI and PII) have been isolated by ethanol precipitation and fractionation on DEAE Sepharose CL-6B and Sephacryl S-400 columns. The structural elucidation was achieved mainly by permethylation, periodate oxidation, basic degradation, methanolysis and reduction experiments, and ^{13}C NMR spectrometry. Both polysaccharides, identified as 4-*O*-methylglucuronoxylans, differ in their M_rs (> 500000 and 40000) only. The polysaccharides show a phagocytosis enhancing effect as determined in three immunological test systems (carbon clearance, granulocyte- and chemiluminescence test).

INTRODUCTION

Extracts of the shrubs *Eupatorium cannabinum* and *Eupatorium perfoliatum* which are widely distributed in Europe and North America respectively, are used in traditional medicine against influenza and other diseases. The chemical investigations of the Eupatoriae species, so far performed, have led to the isolation of sesquiterpene-lactones [1, 2] and highly methoxylated flavonoids [3, 4], which possess antitumor activity as determined in *in vivo* and *in vitro* test systems. These findings do not give a sufficient explanation for the medical use of these drugs. Since recently we have succeeded in the isolation of immunological active polysaccharides from other plants of the Asteraceae [5], we have analysed the polysaccharides of both *Eupatorium* species. The present paper concerns the structure elucidation and the immunological activity of two heteroxylans isolated from *Eupatorium perfoliatum*.

RESULTS AND DISCUSSION

Isolation and purification

Crude polysaccharides were obtained from petrol- and methanol-treated herb material by extraction with 0.5 M sodium hydroxide, followed by precipitation with 3 vols of 95% ethanol. After removal of proteins by trichloroacetic acid the crude polysaccharide mixture was further separated into four fractions by precipitation with increasing concentrations of ethanol (i.e. (a) 1 : 0.5, (b) 1 : 1, (c) 1 : 2 and (d) 1 : 4 respectively). HPLC chromatography of the four fractions on a combined μ-Bondagel-E125 and E500 gelpermeation column showed a very similar composition pattern for both *Eupatorium* polysaccharides. In both cases the fractions a and b represented the major part of the mixtures. Acid hydrolysis (method 1) of both polysaccharides yielded rhamnose, arabinose, xylose, galactose, glucose, 4-*O*-methylglucuronic acid and traces of mannose. The molar sugar ratios of the two first fractions are shown in Table 1. (Biotronic Sugar Analyser, orcein–H_2SO_4). From this it can be seen that also the sugar distribution of the fractions in both polysaccharides is very similar. In particular the precipitates a and b are characterized by a large percentage of xylose.

Since the fraction a of *Eupatorium perfoliatum* exerted the greatest activity in the granulocyte immunotest, all further investigations were performed with this fraction. We fractionated on a DEAE-Sepharose CL-6B column with a 0–1 M sodium chloride gradient and monitored the separation by polarimetric and UV measurements. Rechromatography of the main peak on Sephacryl S 400 with 0.2 M sodium chloride resolved it into two peaks (PI V_e 52 ml; PII V_e 85 ml).

Table 1. Molar sugar composition of the polysaccharide fractions (Biotronic ZA 5100 Sugar Analyser; orcein–H_2SO_4)

Fraction	1:0.5 a	1:0.5 b	1:1 a	1:1 b
Rhamnose	0.40	0.31	0.25	0.18
Arabinose	1.00	1.00	1.00	1.00
Xylose	4.70	16.80	22.00	2.60
Mannose	—	—	0.08	0.11
Galactose	1.70	0.70	1.60	1.14
Glucose	0.40	0.40	0.18	0.34
4-*O*-Methylglucuronic acid	1.60	2.83	0.60	0.66

a, *Eupatorium cannabinum*; b, *Eupatorium perfoliatum*.

*Part II. For part I see Fang, J. N., Proksch, A. and Wagner, H. *Phytochemistry* (in press).

The homogeneity of polysaccharide PII was judged by HPLC, gel chromatography, electrophoresis (one discrete band on cellulose-acetate foil) and ultracentrifugal analysis (one single symmetrical gradient curve). Its M_r was estimated by gel chromatography to be *ca* 40000. The polysaccharide PI was judged to be heteromolecular with an $M_r > 500000$. Both polysaccharides were free of nitrogen.

Structural features of PI and PII

After TFA hydrolysis [7] the polysaccharides yielded (method 2), xylose and 4-*O*-methylglucuronic acid in a molar ratio of about 5.6:1 and traces of arabinose and galactose (< 1%), indicating the presence of 4-*O*-methylglucuronoxylans. After permethylation of PI and PII according to the Hakomori method [8] and conversion into the corresponding partially *O*-methylated alditol acetates 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylxylitol, 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methylxylitol and 1,2,4,5-tetra-*O*-acetyl-3-*O*-methylxylitol were identified by comparing the retention time in GC and mass spectra with those of authentic samples or values in the literature [9]. The measured molar ratios of the three alditol acetates are 0.6:10:3.1 for PI and 0.4:10:2.8 for PII. These results are consistent with a 1→4 linked xylopyranosyl-backbone for both polysaccharides, with a linking point at position C-2 of every sixth or seventh xylose unit. The high negative optical rotation of both polysaccharides and the observed ^{13}C NMR chemical shifts of 104.66 ppm for C-1 and 65.96 ppm for the C-5 signal of the xylopyranosyl residue suggests a β-linkage for the D-xylose units [10]. From the quantitative sugar analysis and the ^{13}C NMR spectroscopy it is estimated that the xylose backbone carries at position C-2 only a single non-reducing 4-*O*-methylglucuronic acid side chain, on average, on every sixth or seventh xylose unit.

The nature of the side chain was investigated by the following experiments: (a) The fully methylated polysaccharide was methanolysed, reduced with $NaBD_4$ and the carboxyl-reduced derivative hydrolysed to give 4-*O*-methylglucose, which was identified by GC/MS [11]. (b) Methanolysis of the methylated polysaccharide followed by acetylation yielded, *inter alia*, anomeric acetylated methyl ester methyl glycosides of the partially methylated aldobiuronic acid (Fig. 1) identified by GC/MS and comparison with a sample similarly generated from methylated 4-*O*-methyl-glucuronoxylan from lucerne [12, 13]. (c) Basic degradation of the permethylated polysaccharides [15] gave no further 3-*O*-methylxylose as could be shown from the GC/MS analysis of the partially methylated and re-deuteromethylated alditol acetates. The molar ratio of 1,4,5-tri-*O*-acetyl-2,3-*O*-methylxylitol to 1,4,5-tri-*O*-acetyl-2-*O*-trideuteromethyl-3-*O*-methylxylitol was *ca* 5:1 indicating that there is no further xylose side chain beyond the single 4-*O*-methylglucuronic acid side chain. This was supported by periodate oxidation which resulted in a molar ratio of unlinked to linked xylose of 5.1:1 for PI and 5.9:1 for PII. The rationalized ^{13}C NMR chemical shifts for the bound 4-*O*-methylglucuronic acid are in full agreement with an α1→2 linkage (Table 2) [16].

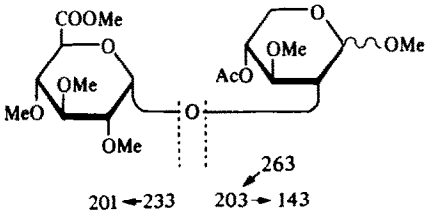
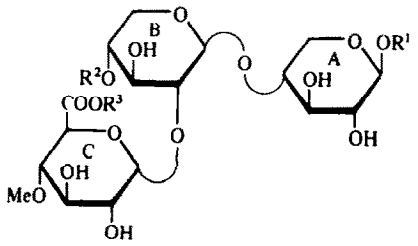


Fig. 1. *M/z* values for the characteristic fragment ions in the mass spectrum of the partially methylated aldobiouronic acid derivatives [12, 14].



From all these results it can be concluded that PI and PII are composed of main units of β-D (1→4) linked xylopyranosyl residues possessing branching points at position C-2 to which 4-*O*-methylglucopyranosyluronic acids are attached as a non-reducing terminal residue on average to one in six or seven of the xylose units.

The unusually large M_r of PI could be explained by association of single xylan chains. This phenomenon has been described several times for xylans [17] and this assumption was supported by two properties of PI:

Table 2. ^{13}C NMR spectral data for PI and PII examined in D_2O

Compound	Ring	Chemical shifts (δ) C-1	C-2	C-3	C-4	C-5	C-6	OMe
	A PI	104.67	75.72	76.72	79.42	66.00		
	PII	104.40	75.43	76.41	79.11	65.71		
	B PI	104.30	79.78	75.33	78.80	—		
	PII	104.00	79.51	75.07	78.78	—		
	C PI	100.56	74.34	75.03	85.33	—	179.62	62.76
	PII	100.53	74.27	75.20	85.36	—	179.90	62.70

R_1: ↳4)-Xyl-(1↳4)-Xyl-(1 . . .

R_2: ↳1)-Xyl-(4↳1)-Xyl-(4 . . . . .

R_3: H

(1) The methylated polysaccharides PI and PII showed the same M_r after gel chromatography on ultragel AcA 202 gel with DMSO as solvent. (2) PI showed in the ultracentrifugal analysis in its main part the same sedimentation-coefficient as PII ($s_0 = 1S$) and only a small part of coefficient 24.5 S. This behaviour can be attributed to a progressive separation of a system of loosely bound clusters from an agglomerate under the increasing centrifugal field, to leave a residue of relatively small M_r [17].

The elucidated structure of this *Eupatorium* polysaccharide is the first one in the Asteraceae family. Similar heteroxylans have been found in the hemicelluloses of hard wood plants and in smaller amounts in soft wood plants [18]. The main differences refer to the ratio of xylose to 4-*O*-methylglucuronic acid, which is mainly reported to be 10:1. In the wood of some Rosaceae plants such as cherry or apple tree, however, other ratios have also been found [19, 20].

Immunological activity

The applied immunological test systems are able to determine the functional state and the efficiency of the human phagocytotic system under the influence of added polysaccharides. The *in vitro* granulocyte tests have been performed with a human granulocyte fraction using yeast particles and opsonized zymosan respectively as challengers. One gives the phagocytic index, the other the yield of chemoluminescence [21, 22]. The *in vivo* carbon clearance [23] determines the elimination rate of carbon particles from the serum on i.v. administrations into mice. The polysaccharide PI enhanced the microphagocytosis at a concentration of 10^{-2}–10^{-5} g/100 ml between 11 up to 30%. The mean yield of chemoluminescence was determined as 20%. These results correlate with the carbon clearance, which revealed a value of RC_{tr}/RC_c of 1.64 (DSK value 2). The polysaccharide PII stimulated the phagocytosis to a smaller extent. The results of investigations on structure activity relationships in the field of polysaccharides of higher plants are published elsewhere [24–26]. It remains to be proved, whether these polysaccharides contribute to the described therapeutic use of the drug.

EXPERIMENTAL

Analytical methods. Optical rotations were measured with a Perkin–Elmer 241 polarimeter. Evaporations were carried out under red. pres. at 40° or less. Dialysis was performed with continual stirring against H_2O at 4°. GC was carried out on a Perkin–Elmer 900 gas chromatograph using a glass column (200 × 0.3 cm) packed with 3% OV 225 on Chromosorb W-HP, 80/100 mesh, 170° for methylation analysis, 150° for glycerol analysis (periodate oxidation analysis), gas flow rate for argon 30 ml/min. R_ts of the partially methylated alditol acetates are given relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol and compared with the lit. [9]. GC/MS: carried out on Carlo Erba (Milano) Fractovap 2110 for split and splitless injection using quartz capillary (30 m × 0.25 mm) coated with OV 1701 interfaced to a Varian CH 7A spectrometer, operating with an ionization potential of 70 eV. Gas flow rate for He: 1.8 ml/min. temp. programme: from 100° to about 260° with 3°/min. Data acquired on a Varian 188-MS data system. The ^{13}CNMR spectrum was determined with a Bruker WP 200 SY, medium D_2O; chemical shifts are given in δ values.

Plant material. Eupatorium cannabinum was collected on the 'Hartsee' (Chiemgau. Oberbayern), air dried and powdered (Voucher No. IR 222). Air dried *Eupatorium perfoliatum* was obtained from Fa. Dr. Willmar Schwabe, Karlsruhe, West Germany and also powdered.

Isolation and purification. The dried powder (1000 g) was first refluxed with petrol (for 2 days) and MeOH (2 days). The residue was extracted with 0.5 M NaOH (10 l.) at 4° overnight and filtered, then washed with 5 l. 0.5 M NaOH. The combined filtrates were precipitated with 45 l. of 95% EtOH, then carefully decanted and centrifuged (15 000 rpm). The ppt. was dissolved in H_2O (3.4 l.) and cold 15% TCA (3.5 l.) was added under stirring; after standing for 1 hr on ice, it was centrifuged. The supernatant was reprecipitated with 24 l. of 95% EtOH, kept for 2 days and then centrifuged, the ppt. dissolved in 2% NaOAc (2 l.) and the insoluble components were filtered. Now the clear soln was precipitated with 8 l. of 95% EtOH standing 4 days at 4°, centrifuged, again dissolved in H_2O (about 200 ml), dialysed for 2 days and lyophilized; yield: *Eupatorium cannabinum*: 1.5%, *Eupatorium perfoliatum*: 2.7% of the dried herb. The obtained crude brownish polysaccharide (20 g) was dissolved in 1 l. H_2O. The solute was precipitated with 0.5 l. of 95% EtOH, and then centrifuged. The same procedure was carried out with the supernatant using 500 ml. 1 l. and 2 l. of EtOH. The precipitations were suspended in H_2O and lyophilized; yield: *E. perfoliatum*: a, 61%; b, 7.5%; c, 16.8%; d, 13.9%. *E. cannabinum*: a, 43%; b, 34.5%; c, 15.5%; d, 10.0%.

(a) *HPLC of the polysaccharides*: 1% solns of polysaccharide fractions in 0.2 M NaOAc buffer pH 4.9 were filtered through Millipore filters (pore size 0.45 μm). HPLC of portions of the filtrates (40 μl) was performed on a Waters chromatograph fitted with μ-Bondagel-E125 and E500, columns (25 × 0.46 cm); flow rate: 0.2–0.4 ml/min, 0.2 M NaOAc buffer, pH 4.9.

(b) *Ion-exchange chromatography*: 200 mg of fraction a of *Eupatorium perfoliatum* was suspended in H_2O (5 ml), centrifuged and applied to a column (60 × 2.6 cm) of DEAE-Sepharose CL-6B. Components on the column were eluted first with H_2O (300 ml), followed by gradient elution with NaCl (0–1 M) and

Table 3. Percentage activation of the microphagocytosis by polysaccharides obtained during isolation PI and PII from the 1:0.5 fraction of *Eupatorium perfoliatum* (in per cent of control)

Fraction	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	g/100 ml
a	0.0%	12.2%	18.4%		no testing		
DEAE-Sepharose							
CL-6b-fraction	7.3%	37.2%	10.0%				
PI	24.5%	29.4%	14.3%	11.2%	2.1%		
PII	0 %	17.2%	22.7%	13.7%	18.6%	4.6%	

Results in per cent of control with 0.9% NaCl solution. Substances are dissolved in 0.9% NaCl. Mean values ($n = 3$).

detected by polarimetric measurement and UV detection at 206 nm. The material giving rise to the main peak a (356–431 ml) was collected, dialysed for 2 days then lyophilized. A white amorphous powder was obtained (yield 35%).

(c) *Gel filtration chromatography*: 100 mg of the above main fraction was dissolved in 0.2 M NaCl (2 ml), applied to a column (60 × 2.6 cm) of Sephacryl S 400 running with 0.2 M NaCl; fractions of 1.5 ml were collected. The two fractions (48–70 ml) and (72–95 ml) corresponding to carbohydrate peaks by determination with refractometer index, optical rotation and UV absorption at 206 nm were collected, dialysed and lyophilized to give PI (yield: 26.8%) and PII (yield: 44.9%).

Determination of homogeneity and molecular weight. Homogeneity was proved by high performance gel permeation chromatography with the μ-Bondagel-E125, E500 and E1000 columns (25 × 0.46 cm; Waters Assoc. Milford, MA, U.S.A. used in HPLC, eluent: 0.2 M NaPi buffer, pH 7) by gel chromatography on Sephacryl S 400 running with 0.2 M NaCl, electrophoresis on cellulose acetate (0.1 N borax/0.1 M NaCl 1:1 pH 9.4), detection: 1% acridine-orange, (5 min, vis) and ultracentrifugal analysis (Type Spinco Modell E. Beckman Instruments) by sedimentation velocity (60000 rpm and 30000 rpm), 0.2% soln in 0.9% NaCl soln.

The M_r calibration curves were performed with dextran T 2000, T 110, T 70 and T 40; a 0.2 M NaCl soln was used as eluent for the Sephacryl S 400 column (60 × 1.6 cm), flow rate was maintained at 14 ml/hr. Peaks were detected by UV absorbance and refractive index.

Analysis of component sugars. Method 1: polysaccharides (1–5 mg) were hydrolysed with 2 M TFA (1–5 ml) at 120° for 1 hr in a sealed tube. The TFA was removed by evaporation under red. pres. The hydrolysate was analysed by a sugar analyser (Biotronic ZA 5100, orcein–H_2SO_4). Method 2: 0.5–2.0 ml TFA was added to the weighed sample (1–5 mg) in a round bottomed flask. The mixture remained overnight at room temp. for swelling or it was heated for 2 hr at 60°; subsequently it was refluxed for 1 hr. The soln was diluted to 80% TFA. After refluxing for 15 min. the soln was further diluted to 30% and again refluxed for 2 hr. After evaporation the dry hydrolysate was analysed by the sugar analyser.

Methylation analysis. Methylation analysis of the polysaccharide was carried out as previously described [8, 9]. The sugar components of the methylated polysaccharide were analysed as their methylated alditol acetates by GC/MS and identified by comparing their retention times and mass spectra with those of authentic samples or with literature values [9].

(a) *Reduction analysis*: 5 mg methylated polysaccharide was methanolized with 3% methanolic HCl for 12 hr at 100°, neutralized with Ag_2CO_3, centrifuged and the supernatant evaporated under red. pres. to dryness, dissolved in 1 ml 2.5% $NaBD_4$ (soln in 95% EtOH) and sonicated for 2 hr. The $NaBD_4$ was destroyed by AcOH (pH 3.5), after distillation (× 3) with 10% AcOH in MeOH the dried residue was hydrolysed (2 M TFA, 100°, 1 hr), then converted into the partially methylated alditol acetates and analysed by GC/MS.

(b) *Partial fragmentation to methylated aldobiuronic acid derivatives*: methylated acidic polysaccharides (5 mg) were methanolysed with 3% HCl in MeOH for 12 hr at 100°, neutralized with Ag_2CO_3 and the resulting methyl glycosides were acetylated (Ac_2O–pyridine, 1:1, 1 hr, 100°) for GC/MS examination.

(c) *Basic degradation (β-elimination of the 4-O-methylglucuronic acid*: methylated polysaccharide (5 mg) was kept in 1 M sodium methylsulphinylmethanide in DMSO at room temp. for 16 hr. CD_3I (1 ml) was added dropwise with cooling and the mixture was stirred at room temp. for 0.5 hr. The degraded methylated PS was isolated in the usual way [9] and the components were analysed by GC/MS of the derived alditol acetates.

Periodate-oxidation. The polysaccharide (12 mg) was oxidized with 0.01 M $NaIO_4$ (20 ml), pH 4, at 4° in the dark. After 7 days the oxidation was stopped, adding 0.4 ml of glycol. After dialysis for 24 hr the non-dialysable fraction was lyophilized, redissolved in 12 ml of H_2O and reduced with $NaBH_4$ (3 mg) for 24 hr at room temp., dialysed and lyophilized, then hydrolysed with 2 M TFA at 100° for 2 hr. Alditol acetate derivatives of the hydrolysate were analysed by GC.

Immunological tests. Granulocyte microscopic and chemiluminescence test and carbon clearance test were performed as previously described [26].

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